Disruption of the p70^{s6k}/p85^{s6k} gene reveals a small mouse phenotype and a new functional S6 kinase

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Recent studies have shown that the p70^{s6k}/p85^{s6k} signaling pathway plays a critical role in cell growth by modulating the translation of a family of mRNAs termed 5'TOPs, which encode components of the protein synthetic apparatus. Here we demonstrate that homozygous disruption of the p70^{s6k}/p85^{s6k} gene does not affect viability or fertility of mice, but that it has a significant effect on animal growth, especially during embryogenesis. Surprisingly, S6 phosphorylation in liver or in fibroblasts from p70^{s6k}/p85^{s6k}-deficient mice proceeds normally in response to mitogen stimulation. Furthermore, serum-induced S6 phosphorylation and translational up-regulation of 5'TOP mRNAs were equally sensitive to the inhibitory effects of rapamycin in mouse embryo fibroblasts derived from p70^{s6k}/p85^{s6k}-deficient and wild-type mice. A search of public databases identified a novel p70^{s6k}/p85^{s6k} homolog which contains the same regulatory motifs and phosphorylation sites known to control kinase activity. This newly identified gene product, termed S6K2, is ubiquitously expressed and displays both mitogen-dependent and rapamycin-sensitive S6 kinase activity. More striking, in p70^{s6k}/p85^{s6k}-deficient mice, the S6K2 gene is up-regulated in all tissues examined, especially in thymus, a main target of rapamycin action. The finding of a new S6 kinase gene, which can partly compensate for p70^{s6k}/p85^{s6k} function, underscores the importance of S6K function in cell growth.

Keywords: cell growth/kinase/rapamycin/S6 phosphorylation/translational control

Introduction

Cell cycle progression in response to mitogens requires the ordered activation of the cyclin-dependent kinase (cdk) family of protein kinases and their cyclin partners (Sherr, 1996). However, for the cell to proliferate, it must also up-regulate the biochemical machinery required to direct cell growth (Nasmyth, 1996). A chief component of the cell growth response is the generation of new translational

machinery (Thomas and Hall, 1997), which is essential for the increased demand of the cell to perform an immense array of distinct anabolic processes (Nasmyth, 1996). Following mitogen-induced exit from G₀, the increased expression of many of the components of the protein synthetic apparatus has been demonstrated to be regulated at the translational level (see Meyuhas *et al.*, 1996). Many of these transcripts are characterized by an oligopyrimidine tract at their transcriptional start site, or 5'TOP (Jefferies *et al.*, 1994a; Terada *et al.*, 1994; Jefferies and Thomas, 1996). Furthermore, recent studies have shown that the translational up-regulation of these transcripts is mediated in part by activation of the p70^{s6k}/p85^{s6k} (Jefferies *et al.*, 1997), presumably through 40S ribosomal protein S6, whose increased phosphorylation is hypothesized to facilitate the recognition of these transcripts by the 43S pre-initiation complex (Jefferies *et al.*, 1994a). The p85^{s6k} isoform is expressed from the same transcript as p70^{s6k}, through an alternative translational initiation start site (unpublished data), which adds a 23 amino acid nuclear localization signal (NLS) to the N-terminus (Reinhard *et al.*, 1994). The importance of these two kinases in cell growth has been inferred from either the use of the immunosuppressant rapamycin (Chung *et al.*, 1992; Kuo *et al.*, 1992; Price *et al.*, 1992) or through microinjection of neutralizing antibodies into cells (Lane *et al.*, 1993; Reinhard *et al.*, 1994), both of which selectively suppress mitogen-induced p70^{s6k}/p85^{s6k} activation and impede cell growth.

The signal transduction pathway which mediates p70^{s6k}/p85^{s6k} activation has attracted a considerable degree of attention, because of its implied importance in cell growth and its potential use in identifying novel targets for immunosuppressive therapy (see Downward, 1998; Peterson and Schreiber, 1998). This pathway bifurcates at a growth factor receptor docking site that is distinct from that of the Ras–MAP kinase pathway (Ming *et al.*, 1994), with activated phosphatidylinositol-3 kinase (PI3-K) as the proximal signaling component initiating downstream signaling (Chung *et al.*, 1994). However, not all the data are consistent with this model nor with the role of other potential signaling components implicated in regulating p70^{s6k}/p85^{s6k} activation (see Downward, 1994, 1995; Thomas and Hall, 1997). A large part of this uncertainty stems from the complex nature of the p70^{s6k}/p85^{s6k} activation event, which relies on the sequential interplay between multiple phosphorylation sites (Ferrari *et al.*, 1992; Pearson *et al.*, 1995; Moser *et al.*, 1997) and distinct intramolecular regulatory domains within the kinase (Pullen and Thomas, 1997; Dennis *et al.*, 1998). Rapamycin blocks p70^{s6k}/p85^{s6k} activation through inhibition of mTOR/FRAP, a large molecular weight protein which is thought to serve as either a lipid or protein kinase (Thomas and Hall, 1997; Peterson and Schreiber, 1998). As treatment of cells

Fig. 1. Molecular analysis of $p70^{s6k}$ -deleted mice. (A) $p70^{s6k}$ gene map and targeting vector: the exons containing the T loop or APE sequence are shown as closed or hatched boxes, respectively. (B) Southern blot analysis of DNA from $p70^{s6k+/+}$ (+/+), $p70^{s6k+/-}$ (+/-) and $p70^{s6k-/-}$ (-/-) mice, following *Bam*HI digestion and employing the radioactive probe indicated in (A). Closed arrow shows bands corresponding to the wild-type allele and the open arrow shows those corresponding to the targeted allele. (C) Western blot analysis of total thymus protein extract using either the M1 or M5 antibody, directed against a peptide corresponding to either the C- or N-terminus of $p70^{s6k}$, respectively. The position of $p70^{s6k}$ is indicated and lanes are labeled as in (B). (D) Total liver extracts from mice intraperitoneally injected with cycloheximide (5 mg/100 g body weight) were immunoprecipitated with either the M5 or M1 antibody and subjected to an S6 kinase assay (Materials and methods).

with rapamycin selectively blocks $p70^{s6k}/p85^{s6k}$ and S6 phosphorylation without affecting other protein kinases, it has been argued that $p70^{s6k}/p85^{s6k}$ is the sole *in vivo* kinase responsible for regulating S6 phosphorylation. This conclusion was substantiated by the use of rapamycin-resistant $p70^{s6k}/p85^{s6k}$ mutants which protected S6 dephosphorylation from the bacterial macrolide (von Manteuffel *et al.*, 1997). The potential impact of rapamycin treatment in immunosuppressive therapy combined with the importance of delineating mTOR/FRAP function has stimulated a great deal of interest in $p70^{s6k}/p85^{s6k}$ (Thomas and Hall, 1997).

Although studies with rapamycin as well as inhibitory antibodies have led to the hypothesis that $p70^{s6k}/p85^{s6k}$ plays an essential role in cell growth (Chou and Blenis, 1995), corroborative physiological studies have been lacking. Recently, however, we have identified a P-element-induced mutation in the *Drosophila melanogaster* homolog of the *dp70s6k* gene which severely impairs kinase expression and cell growth (unpublished data). Nevertheless, there is greater genetic diversity in mammals than in lower metazoans, and in many cases functional redundancy exists which is not revealed in less complex organisms (Miklos and Rubin, 1996). For these reasons, we set out to disrupt the $p70^{s6k}/p85^{s6k}$ gene from mouse to evaluate its functional uniqueness in higher organisms and to determine which of the effects of rapamycin on cell growth are elicited through inhibition of $p70^{s6k}/p85^{s6k}$ activity and S6 phosphorylation.

Results

Disruption of the S6 kinase gene

Screening a mouse genomic library with a probe corresponding to the catalytic domain of a rat $p70^{s6k}/p85^{s6k}$ cDNA (Kozma *et al.*, 1990) resulted in the isolation of a 14 kb fragment of the mouse $p70^{s6k}$ gene (Materials and methods). To create a gene disruption by homologous recombination (Capecchi, 1989), we generated a targeting

construct by replacing 1.2 kb of the genomic sequence encompassing the conserved Ser/Thr kinase catalytic subdomains VIII–X, with a neomycin resistance cassette (Figure 1A). Of 316 embryonic stem (ES) cell clones, three harbored the expected targeted mutation, as identified by PCR and Southern blot analysis (data not shown). One positive clone was used to establish chimeric mice that transmitted the targeted mutation through the germ line (Wood *et al.*, 1993). Hetero- and homozygous mutant mice (Figure 1B) were obtained in Mendelian proportions, suggesting no obvious effect on viability. To confirm that the gene had been disrupted, Western blot analysis was performed with two distinct $p70^{s6k}$ antibodies which recognize either the N- or C-terminus of $p70^{s6k}$ (Figure 1C). The results revealed a 50% decrease of the signal in heterozygous mice and no apparent signal in homozygous mice (Figure 1C). Similar results were obtained for the $p85^{s6k}$ isoform (data not shown). To test for S6 kinase activity, wild-type, heterozygous and homozygous animals were injected intraperitoneally with cycloheximide, a treatment known to induce $p70^{s6k}$ activation in liver (Kozma *et al.*, 1989). Liver extracts from these mice were assayed for $p70^{s6k}$ kinase activity by immunoprecipitation with either of the two antibodies described above. The S6 kinase phosphorylation assay revealed reduced activity in liver extracts from heterozygous as compared with wild-type mice and no activity from homozygous mutant mice (Figure 1D). These results showed that the $p70^{s6k}/p85^{s6k}$ gene had been disrupted and that the activity corresponding to this protein was absent from the homozygous mutant mice.

Phenotype of $p70^{s6k}$ gene disruption in mice

Three-week-old homozygous mutant mice were significantly reduced in body size when compared with wild-type mice (Figure 2A). To ensure there were no variations due to *in utero* competition, body weights obtained at birth from either homozygous mutant or wild-type crosses were compared (Table I). In both cases, the average body weight

Fig. 2. Mice lacking the $p70^{s6k}$ gene exhibit a small size phenotype. (A) Photograph of $p70^{s6k+/+}$ (+/+) and $p70^{s6k-/-}$ (-/-) littermates at 3 weeks of age. (B) Mean organ weights from three $p70^{s6k-/-}$ male animals at the age of 3.5 weeks. Results are plotted as the percentage in weight of $p70^{s6k-/-}$ mice compared with three wild-type mice. (C) Mean body weight of 20 $p70^{s6k+/+}$ (+/+) and $p70^{s6k-/-}$ (-/-) male mice, and $p70^{s6k+/+}$ (+/+) and $p70^{s6k-/-}$ (-/-) female mice. Samples are siblings from 23 pairs of heterozygous mating.

difference was ~20% for both females and males. A comparison of homozygous mutant mice at 3.5 weeks of age demonstrated that the weights of all organs were proportional to the reduction in body weight (Figure 2B). Heterozygous mice were slightly smaller, although the values obtained were not significantly different from those obtained for wild-type mice (data not shown). Analysis of body weight over an 11 week period following birth (Figure 2C) showed that up to ~5 weeks of age both homozygous males and females grew more slowly than their wild-type littermates. However, between 5 and 6 weeks of age, the growth rate for both homozygous and wild-type offspring dramatically slowed down (Figure 2C),

Table I. Body weights (g) at birth from either homozygous mutant or wild-type crosses

	Male	Female
+/+	1.72 ± 0.07 (n = 7)	1.62 ± 0.16 (n = 5)
-/-	1.42 ± 0.10 (n = 9)	1.32 ± 0.09 (n = 3)

Values represent the mean and the standard deviation. *n*, number of animals.

after which it appeared to be equivalent up to 11 weeks. The difference in body weight for both males and females was ~15% at this stage. Thus disruption of the $p70^{s6k}/p85^{s6k}$ gene resulted in a reduction in body weight and smaller animals.

Growth of embryos and primary embryo fibroblasts

As homozygous animals appeared to be retarded in their growth, embryos were analyzed at different stages *in utero*. The results revealed no obvious morphological differences between homozygous mutants and wild-type animals at any stage of development. However, as depicted in a typical litter at 14.5 days of gestation, homozygous mutants were distinctly smaller in size, suggesting a growth delay without a corresponding delay in the rate of development. The difference in weight can be >30%, with heterozygotes displaying intermediate values (Figure 3A). To determine whether the effects on size were due to an impeded rate of cellular proliferation, 13.5 day mouse embryo fibroblasts (MEFs) from homozygous mutants and wild-type animals were tested for their ability to proliferate in culture. Under the standard culture conditions employed, there was no difference in the ability of the two cell types to proliferate (Figure 3B). Furthermore, growth inhibition by rapamycin was similar in MEFs from both homozygous mutant and wild-type mice (Figure 3C), suggesting that there was an additional target by which rapamycin exerted its inhibitory effects on cell growth in fibroblasts. Indeed, analysis of polysome profiles showed that loss of $p70^{s6k}/p85^{s6k}$ does not affect the ability of mitogens to up-regulate the expression of eEF1- α , a typical 5'TOP mRNA (Figure 3D). Furthermore, rapamycin suppressed the up-regulation of eEF1- α to the same extent in MEFs derived from wild-type and homozygous mutant animals (Figure 3D). Thus, the difference in embryo size was not reflected in the rate of MEF proliferation neither the effects of rapamycin on cell proliferation nor the translation of 5'TOP mRNAs could be attributed solely to $p70^{s6k}/p85^{s6k}$.

S6 phosphorylation in $p70^{s6k}$ -deficient mice

Despite earlier studies indicating that $p70^{s6k}/p85^{s6k}$ plays a critical role in the translation of 5'TOP mRNAs (Jefferies *et al.*, 1997), there was no difference in this response in $p70^{s6k}/p85^{s6k}$ -deficient mice (Figure 3D). The effects of $p70^{s6k}$ on this response as well as on cell growth have been hypothesized to be mediated through increased S6 phosphorylation, and studies with rapamycin or dominant-negative mutants of $p70^{s6k}$ have led to the conclusion that it was the sole kinase responsible for mediating this event *in vivo* (Chung *et al.*, 1992; Jefferies *et al.*, 1994a;

Fig. 3. Effect of the $p70^{\text{s6k}}$ -deleted gene on embryos and mouse embryo fibroblasts. **(A)** Photograph of $p70^{\text{s6k}+/+}$, $p70^{\text{s6k}+/-}$ and $p70^{\text{s6k}-/-}$ male embryos at day 14.5. The sex of the embryos was identified by PCR based on sequences specific to Y chromosome (Voss *et al.*, 1997). The body weights of $+/+$, $+/-$ and $-/-$ embryos were 182, 168 and 125 mg, respectively. **(B)** Proliferation and saturation density of MEFs from $p70^{\text{s6k}+/+}$ (□) and $p70^{\text{s6k}-/-}$ embryos (◆). Cells were plated in a 6-well plate and counted, as described in Materials and methods. Each value is an average of duplicate plates. The experiment was repeated twice with reproducible results. **(C)** Effect of rapamycin on the cell proliferation. Cells were plated in 24 wells as described in Materials and methods. Each value is an average of triplicate plates. The experiment was repeated three times with reproducible results. **(D)** The translation of 5'TOP mRNAs is up-regulated by mitogens and sensitive to rapamycin in MEFs derived from $p70^{\text{s6k}-/-}$ animals. Cytoplasmic extracts were prepared from $p70^{\text{s6k}+/+}$ ($+/+$) or $p70^{\text{s6k}-/-}$ ($-/-$) MEFs that were starved in 0.5% serum for 48 h or stimulated for 4 h with 10% FCS after starvation, either directly or after a pre-treatment for 15 min with 20 nM rapamycin. The extracts were centrifuged on a 17.1–51% sucrose gradient, fractionated and the fractions analyzed by Northern blot using specific probes for eEF-1 α or actin mRNAs. The blots were visualized by PhosphorImager (Molecular Dynamics). The figure shows the relative amount of mRNA present in each fraction expressed as a percentage, as calculated using ImageQuant software (Molecular Dynamics). Fractions 1–7 contain polysomes whereas fractions 8–12 are enriched in monosomes, ribosomal subunits and mRNPs.

von Manteuffel *et al.*, 1997). To ensure that this response was abrogated in $p70^{\text{s6k}}/p85^{\text{s6k}}$ -deficient mice, the extent of S6 phosphorylation was examined in the livers of wild-type and homozygous mutant mice following re-feeding of starved animals, a protocol which previously had been shown to induce $p70^{\text{s6k}}$ activation and S6 phosphorylation in rats (Kozma *et al.*, 1989). Two-dimensional polyacrylamide gel analysis revealed that in starved wild-type or mutant mice, S6 resided in the unphosphorylated form (Figure 4A), with only small amounts of the singly and doubly phosphorylated derivative, S6a and b, present. Re-feeding of wild-type mice induced a large increase in S6 phosphorylation (Figure 4A), with the appearance of all five of the phosphorylated S6 derivatives, S6a–e, clearly detectable. Surprisingly, in the liver of $p70^{\text{s6k}}/p85^{\text{s6k}}$ -deficient animals, the extent of S6 phosphorylation was comparable (Figure 4A). Similar results were also obtained if S6 phosphorylation was induced by intraperitoneal injection

of cycloheximide (data not shown), which is thought to trigger increased S6 phosphorylation through inhibition of protein synthesis (Blenis *et al.*, 1991). Consistent with these observations, serum stimulation of MEFs from either wild-type or $p70^{\text{s6k}}/p85^{\text{s6k}}$ -deficient mice also induced full phosphorylation of the ribosomal protein, with most of S6 migrating in the position of the most highly phosphorylated derivatives S6d and e (Figure 4B). Furthermore, rapamycin pre-treatment abrogated this response in MEFs from both wild-type and $p70^{\text{s6k}}/p85^{\text{s6k}}$ -deficient mice (Figure 4B). The results demonstrated that in response to either re-feeding or mitogen stimulation, S6 phosphorylation in the tissues examined was unimpaired in $p70^{\text{s6k}}/p85^{\text{s6k}}$ -deficient animals and remained sensitive to rapamycin.

A novel rapamycin-sensitive S6 kinase activity

The results above implied that the mouse genome contained an unidentified rapamycin-sensitive kinase capable

Fig. 4. S6 phosphorylation in p70^{s6k}-deleted mice. (A) S6 phosphorylation in liver. Two-dimensional gel electrophoresis of 80S ribosomal proteins from p70^{s6k}/p85^{s6k}+/+ (left panels) or p70^{s6k}/p85^{s6k}-/- mice (right panels). Mice were starved for 24 h and re-fed for 1 h. (B) Inhibition of S6 phosphorylation by rapamycin. Two-dimensional gel electrophoresis of 80S ribosomal proteins from p70^{s6k}/p85^{s6k}+/+ (+/+) or p70^{s6k}/p85^{s6k}-/- (-/-) MEFs. Cells were stimulated with 10% FCS for 1 h with or without treatment with 20 nM rapamycin. Rapamycin was added for 15 min prior to the addition of serum.

of inducing S6 phosphorylation. To test this possibility, extracts from serum-stimulated MEFs derived from wild-type and p70^{s6k}/p85^{s6k}-deficient mice pre-treated in the absence or presence of rapamycin were analyzed by Mono S chromatography. Extracts from wild-type mice showed a major peak of S6 kinase activity which eluted at ~0.25 M NaCl and a less pronounced peak at 0.45 M (Figure 5A). Pre-treatment of cells with rapamycin abolished both peaks of activity (Figure 5A). Previous studies had shown that the first peak represented the p70^{s6k} (Jenö *et al.*, 1988) and, as expected, the first peak of S6 kinase activity was absent in extracts from MEFs of p70^{s6k}/p85^{s6k}-deficient mice (Figure 5A). However, the second peak of activity did not disappear and seemed to be slightly elevated in extracts from p70^{s6k}/p85^{s6k}-deficient mice relative to wild-type mice (Figure 5A), suggesting that the gene product may be up-regulated in mice lacking the p70^{s6k}/p85^{s6k} gene. These results strongly indicated the presence of a novel and as yet unidentified rapamycin-sensitive S6 kinase. Based on these observations, a search of the existing expressed sequence tag (EST) databases was initiated (Bugowski *et al.*, 1993), resulting in the identification of a putative homolog of p70^{s6k}/p85^{s6k} (Figure 5B). We termed this homolog S6 kinase 2, (S6K2), and for clarity have termed p70^{s6k}, S6K1 (Figure 5C). A comparison of the S6K1 sequence with that of S6K2 reveals that they were highly homologous, sharing 82% identity in the catalytic domain (Figure 5C). In addition, all the important regulatory domains are conserved, including the acidic N-terminus, the linker region and the autoinhibitory domain, as well as the critical phosphoryl-

ation sites, including those equivalent to T229, S371, T389 and the S/TP sites in the autoinhibitory domain of S6K1 (Pearson *et al.*, 1995; Moser *et al.*, 1997). Clearly absent was the 23 amino acid NLS found in the p85^{s6k} isoform (Reinhard *et al.*, 1994), although a potential NLS resides at the C-terminus of S6K2 (Figure 5B). Thus, the existence of a novel S6K was consistent with the regulated S6 phosphorylation identified in S6K1-deficient mice.

Identification of S6K2

To determine whether the second peak of S6 kinase activity observed in extracts from wild-type MEFs (Figure 5A) eluted in the same position as S6K2, extracts were prepared from serum-stimulated 293 control cells or 293 cells that had been transiently transfected with a myc epitope-tagged S6K2. The extracts were then analyzed by Mono S column chromatography as described above. The data showed that in comparison with extracts from control cells, the later eluting peak of S6 kinase activity was greatly enhanced in extracts containing the epitope-tagged kinase (Figure 6A), in agreement with this peak representing S6K2. In addition, insulin-induced activation of the transiently expressed myc-S6K2 in 293 cells and this response was blocked by rapamycin (Figure 6B), consistent with the data presented above for MEF extracts from p70^{s6k}-deficient mice (Figures 4B and 5A). To ensure that the second peak represented S6K2, the equivalent Mono S peak fractions from liver extracts of p70^{s6k}/p85^{s6k}-deficient mice, which had been subjected to cycloheximide treatment, were concentrated on an S6-peptide affinity column (Materials and methods). This material was then analyzed for kinase activity and for the presence of S6K2 employing an antiserum directed against an N-terminal sequence of S6K2 (Materials and methods). This antiserum, as compared with pre-immune serum, specifically immunoprecipitated S6 kinase activity and detected a single band on Western blots which migrated at the predicted molecular weight of S6K2 (Figure 6C). Immunodepletion studies showed further that most of this activity could be removed from the pooled fraction after three successive immunoprecipitations, suggesting the existence of a single kinase (data not shown). Finally, RNase protection studies revealed that S6K2 transcripts appear to be ubiquitously expressed and increased in all tissues examined in S6K1-deficient versus wild-type mice (Figure 6D). The up-regulation of S6K2 is especially high in thymus, where it reaches 3.8-fold the level observed in wild-type animals when normalized to a control mRNA (Figure 6D). These results suggested that in mice lacking S6K1, a compensatory mechanism leads to higher expression of S6K2, especially in the thymus where rapamycin had been shown to have a more pronounced effect on cell growth (Luo *et al.*, 1994).

Discussion

Here we demonstrate that S6K1-deficient mice are viable and fertile, but they are significantly smaller in size (Figure 2), a phenotype particularly obvious in embryos (Figure 3). Surprisingly, the S6 phosphorylation response was unimpaired in livers of S6K1-deficient mice following either a starvation/re-feeding regime or injection with cycloheximide (Figure 4A). Furthermore, MEFs derived

Fig. 5. Existence of a novel S6 kinase. (A) Elution profile of S6 kinase activity from a Mono S column. $p70^{s6k+/+}$ (+/+) and $p70^{s6k-/-}$ (-/-) MEFs were stimulated with 10% FCS for 1 h with (□) or without (◆) pre-treatment (15 min) with 20 nM rapamycin. Equal amounts of cell extracts (6 mg) were fractionated on a Mono S column using a salt gradient from 0 to 0.5 M NaCl. The two peaks of activity present in serum-stimulated wild-type MEFs eluted at ~0.25 and 0.45 M NaCl. (B) Schematic diagram of S6K2 showing the N-terminus domain (cross-hatched), the catalytic domain (open), the linker domain (hatched), the autoinhibitory domain (filled) and the C-terminus domain (dotted). Phosphorylation sites that are conserved with $p70^{s6k}$ are indicated. The acidic sequence, the proline-rich sequence and a putative NLS are also indicated. (C) Amino acid sequence alignment of S6K1 and S6K2. Identical amino acids are indicated by a bar, similar amino acids are indicated by dots.

from S6K1-deficient mice, as compared with wild-type mice, displayed an equivalent sensitivity to rapamycin in terms of serum-induced proliferation, 5'TOP mRNA translation and S6 phosphorylation (Figures 3 and 4B). These findings led to the identification of a new S6 kinase that functionally overlaps with S6K1 and whose transcripts are up-regulated in S6K1-deficient mice (Figure 6D).

Despite the presence of S6K2, S6K1-deficient mice are smaller in size. This result provides further evidence for an involvement of the PI3-K/S6K signal transduction pathway in growth control. It was already shown that disruption of upstream signaling components, such as the IGF receptor 1 (J.-P.Liu *et al.*, 1993) and the insulin receptor substrate 1 and 2 (IRS1 and 2) (Araki *et al.*,

Fig. 6. Functional characterization and mRNA expression of S6K2. (A) Elution profile of S6 kinase activity from a Mono S column. Insulin-stimulated 293 cells were transiently transfected with vector DNA (□) or with 2 μ g of myc-S6K2 expression plasmid (◆). Equal amounts of cell extracts (6 mg) were fractionated on a Mono S column using a salt gradient from 0 to 0.5 M NaCl. (B) Inhibition of S6K2 activity by rapamycin. 293 cells overexpressing myc-S6K2 were either left untreated or treated with 20 nM rapamycin, or stimulated with insulin in the absence or presence of rapamycin, as indicated. Equal amounts of cell extracts (20 μ g) were immunoprecipitated using 9E10 antibody, and S6 kinase activity of the immune complex was assayed (top panel). The same amount of cell extracts was analyzed by Western blot using 9E10 antibody (bottom panel). (C) S6K2 activity is present in S6K1-deficient mice. S6K1-deficient (–/–) mice were injected with cycloheximide (5 mg/100 g body weight). After 1 h, mice were sacrificed and liver extracts prepared. The extract (15 mg of proteins) was subjected to Mono S chromatography as in (A), the fractions assayed for S6 kinase activity, and the active fractions were pooled (19–26) and concentrated on an S6 peptide affinity column (Materials and methods). This material was either assayed in an immune complex assay for S6K2 activity (top panel) or analyzed for the presence of the kinase by Western blot analysis (bottom panel) employing an antiserum against S6K2. Apparent molecular weights are indicated. (D) S6K2 mRNA levels are up-regulated in S6K1-deficient mice. Total RNA (20 μ g) from wild-type (+/+) and S6K1-deficient (–/–) mouse liver, muscle, thymus and brain were analyzed by RNase protection assay using a S6K2-specific probe and cytochrome oxidase 1 probe as internal control for RNA recovery. S6K2 levels were quantified by phosphorimager and normalized with the levels of cytochrome oxidase 1. The fold induction of S6K2 expression in S6K1-deficient mice is indicated.

1994; Tamemoto *et al.*, 1994; Withers *et al.*, 1998), affect animal size. Moreover, in *Drosophila*, ectopic expression of PI3-K recently has been demonstrated to promote

growth of tissues by positively regulating both cell size and cell number (Leever *et al.*, 1996). It is clear that several signaling pathways, in addition to that of S6K, originate from the growth factor receptor–PI3-K complex and are likely to cooperate in the control of growth. In the case of S6K1, the small size of homozygous mutant mice is consistent with a defect in translational capacity. Mutants have been described in *Drosophila* in which either expression of ribosomal proteins or rRNA are affected, termed *Minutes* or *bobbed* (Shermoen and Kiefer, 1975; Procunier and Dunn, 1978; Lindsley and Zimm, 1992). Either set of mutations leads to smaller flies which are delayed in development, similar to that reported here for S6K1-deficient mice. Consistent with the role of S6K1 in translation, a P-element insertion in the 5' untranslated region (5'UTR) of the *Drosophila* homolog of the S6K gene, which severely suppresses the expression of the kinase, induces a *Minute*-like phenotype (unpublished data). As S6K1 has been shown to be involved in regulating the expression of 5'TOP mRNAs at the translational level (Jefferies *et al.*, 1997), a deficiency in the gene product would be expected to lead to reduced translational capacity and, consequently, the generation of smaller animals. Thus, it may be that in certain cell types or during specific development stages, the newly described S6K2 cannot fully compensate for the loss of S6K1 function with respect to S6 phosphorylation and the up-regulation of 5'TOP mRNAs. To address this issue, a thorough analysis of these responses in specific organs and tissues throughout development will be required. In addition, the relative contributions of both genes in individual tissues would be greatly facilitated by generating mice containing a deletion of the S6K2 gene, as well as mice containing deletions of both genes.

Previous biochemical studies (Jenö *et al.*, 1988) or extensive screening of cDNA libraries gave no indication of a second S6 kinase (Banerjee *et al.*, 1990; Kozma *et al.*, 1990; Grove *et al.*, 1991). In addition, despite the high level of identity between S6K1 and S6K2, antibodies generated against S6K1 do not cross-react with S6K2 (Figure 1). In most cases, S6K1 activity has been analyzed by Mono Q chromatography (Jenö *et al.*, 1988). Under these conditions, we have found that both kinases eluted in the same fraction (data not shown). Furthermore, in the initial purification of S6K1, Mono S chromatography was not employed until the final step (Jenö *et al.*, 1988, 1989); at this point S6K2 may have been separated from the S6K1 active material. This fact, along with preliminary data showing that S6K2 is more sensitive to phosphatases than S6K1 during extraction (M.Sanders and M.Pende, unpublished), may explain why S6K2 was not detected in earlier biochemical studies. Here we show that Mono S chromatography of MEFs and 293 cell extracts reveals both peaks of S6K activity. The major peak of activity corresponds to S6K1, and the second peak represents S6K2. These data, together with extensive searches of EST databases, suggest that there are no additional homologs of functional S6 kinases in mice. It also appears from the Mono S chromatography profile that S6K1 is the more dominant activity; however, the *in vitro* assay conditions employed may not be optimal for detection of S6K2 activity. Furthermore, as stated above, S6K2 appears to be more sensitive to phosphatases. Higher *in vivo* S6K2

activity might explain the robust S6 phosphorylation response observed in the livers or fibroblasts derived from S6K1-deficient mice. It is also clear that S6K2 transcripts are up-regulated in S6K1-deficient mice (Figure 6D), and the same may be true for protein levels; however, to resolve this latter point will require higher affinity antibodies. Another possibility is that protein phosphatase 1, which selectively dephosphorylates S6 *in vitro* (Olivier *et al.*, 1988), could also be suppressed in S6K1-deficient mice, compensating for the loss of S6K1 and leading to higher levels of S6 phosphorylation. Evidence for such a mechanism has been demonstrated in cells transformed by temperature-sensitive Src, where high levels of S6 phosphorylation are largely maintained through suppression of protein phosphatase 1 activity rather than S6K activation (Belandia *et al.*, 1994). Future studies employing the use of high affinity S6K2 antibodies as well as assays designed to measure the impact of protein phosphatase 1 on the S6 phosphorylation response will be important in resolving whether such compensatory mechanisms are operating in S6K1-deficient mice.

The identity between S6K1 and S6K2 is >80% in the catalytic domain, suggesting that these forms result from gene duplication of the type described for Hox and tyrosine kinase genes in vertebrates (Spring, 1997; Aparicio, 1998). The high degree of S6K1/S6K2 identity in the catalytic domain extends to the linker region where critical phosphorylation sites involved in regulating S6 kinase activity reside, including S370 and T388. Indeed, of the eight phosphorylation sites identified in endogenous S6K1, all, as well as their surrounding motifs, are present in S6K2 (Figure 5). In addition to these sites, phosphorylation of S17 (Weng *et al.*, 1995) as well as of T367 and T447 (Pearson *et al.*, 1995) has been identified in overexpressed S6K1. Of these sites, only T447 is absent in S6K2. The homologous site to T388, T389 in S6K1, is the principle target of rapamycin-induced dephosphorylation and inactivation of S6K1 (Pearson *et al.*, 1995). This effect requires the acidic N-terminus of S6K1 (Dennis *et al.*, 1996), which is also present in S6K2, consistent with the fact that S6K2 is also sensitive to rapamycin (Figure 6B). Noticeably absent in S6K2 is evidence of a nuclear targeting sequence, such as that found in S6K1. However, as the 5'UTR of the S6K2 gene does not reveal in-frame stop codons, a nuclear-targeted S6K2 isoform could still be generated by an alternative translational start site at an upstream codon. In addition, there is a basic sequence at the C-terminal end of S6K2 which could also serve as an NLS (Figure 5B). Additional studies employing better antibodies to the endogenous kinase will be required to resolve this issue. The other striking feature of S6K2 is a sequence extending from residue 435 to 459, which contains 50% prolines and is noticeably absent in S6K1. A search of potential related sequences showed that residues 453–469 are quite homologous to a domain in the p85 subunit of the PI3-K which has been shown to bind to Src kinase (X.Liu *et al.*, 1993). If this motif acts as a docking site for an SH3-containing protein, it would suggest a role for S6K2 distinct from that of S6K1 in downstream signaling.

During the completion of these studies, it was reported that deletion by homologous recombination of the S6K1 gene in ES cells led to the generation of a clonal line

which exhibited a 50% slower growth rate and maintained rapamycin sensitivity (Kawasome *et al.*, 1998). In this case, no phosphorylation of S6 was detected in homozygous deleted S6K1 cells versus either wild-type or heterozygous cells. Furthermore, 5'TOP transcripts were present on polysomes in the absence of mitogens, whereas these transcripts were largely present in mRNP particles in wild-type cells, as previously reported (Jefferies *et al.*, 1994a; Terada *et al.*, 1994; Jefferies and Thomas, 1996). The slower growth rate of these embryonic stem cells could be viewed as being consistent with the apparently slower rate of growth exhibited by S6K1 embryos. Although the initial S6K2 EST was derived from a mouse blastocyst library (Lennon *et al.*, 1995) and we have detected S6K2 transcripts in every other cell type we have examined, it may be that S6K2 is non-functional in ES cells. However, it should also be noted that the method employed by Kawasome *et al.* (1998) to examine S6 phosphorylation was ³²P-labeling of growing cells. Under these conditions, S6 phosphorylation is normally quite low (Nielsen *et al.*, 1981). Future studies should resolve these differences.

In most cell types, rapamycin delays cell proliferation whereas in T cells it abolishes this response (Kuo *et al.*, 1992); this effect is also observed *in vivo* (Luo *et al.*, 1994). This selectivity for T cells is being exploited in immunosuppressive therapies following organ transplantation (Borman, 1994). We have found that the level of S6K1 is particularly high in thymus. However, we have seen no difference in T cell proliferation induced by co-stimulation with CD28/CD3 antibodies in cells derived from either S6K1-deficient or wild-type mice (unpublished data). Nevertheless, the extent to which S6K2 transcripts are up-regulated in S6K1-deficient mice is most dramatic in thymus, suggesting an important role for S6K in this organ. Disruption of the S6K2 gene in the S6K1-deficient background and the use of RAG-2 mice (Chen, 1993) should resolve the question of the importance of S6K in T cell proliferation and its value as a potential therapeutic target for transplantation therapy.

Materials and methods

Construction of the targeting vector

By screening a 129/SV mouse genomic library (Stratagene) with p70^{S6K}cDNA as a probe, we obtained a 14 kb fragment that contained the sequence of the catalytic domain of p70^{S6K}. The 3.4 kb *NheI*–*ApaI* fragment and the 1.0 kb *SpeI*–*PstI* fragment of the p70^{S6K}/p85^{S6K} gene were ligated into the *XbaI* and *Sall* sites of the neo cassette vector (Arber *et al.*, 1997), respectively. The resulting targeting vector was designated pS6Kneo/TK.

Electroporation of embryonic stem cells

The pS6Kneo/TK targeting vector (50 µg) was linearized at the single *NotI* site and electroporated into 1.0×10⁷ E14 ES cells at 500 mF and 250 V. The cells were then cultured in the presence of G418 (0.6 mg/ml) and gancyclovir (2 µM) for 10 days. Homologous recombination events were identified by PCR. The forward primer for the PCR was 5'-TGCTCTCAGTCATGGTCTCCACCAA, which corresponds to the sequence of the 3' end of the neo cassette. The reverse primer was 5'-AACGCACGGGTGTTGGGTCGTTTG, which corresponds to the sequence of the p70^{S6K} gene flanking the short arm of the targeting vector. The primers were used according to the following cycling conditions: 3 min at 94°C (one cycle); 0.5 min at 94°C, 0.3 min at 67°C and 1 min at 72°C (40 cycles); 10 min at 72°C (one cycle). ES cell colonies were screened by PCR for the 1.1 kb fragment specific of the targeting event.

Chimera generation and Southern blot analysis

Aggregation of positive ES cells with morula-stage embryos obtained from inbred (C57Bl/6 $\times$ DBA/2) F₁ mice was performed following Wood *et al.* (1993). Chimeric mice were crossed to C57Bl/6 mice and germline transmission was assessed by Southern blot analysis. DNA extracted from the tails of mice was digested with *Bam*HI, and 10 μ g was loaded onto a 0.7% agarose gel and transferred to a nylon membrane (Porablot, Macherey-Nagel). Hybridization and washing were performed as previously described (Reinhard *et al.*, 1992). The probe used for hybridization was the 300 bp PCR product amplified with the forward primer, 5'-ACAGTGGATGAGCCTACTTG, and the reverse primer, 5'-GGA-CGGAGGTATCAGTGTAG.

Isolation of mouse embryo fibroblasts, cell cultures and two-dimensional gel electrophoresis

Day 13.5 embryos were minced and incubated in 0.25% trypsin at 37°C for 15 min, and then filtered through a cell strainer (Falcon) as previously described (Blasco *et al.*, 1997). Dulbecco's modified Eagle's medium (DMEM), containing fetal calf serum (FCS) at a final concentration of 10%, was then added to the cell suspension. Cells were then centrifuged at 1000 r.p.m. and the pellet was suspended in DMEM containing 10% FCS. To measure cell doubling times and saturation densities, cells were plated in 6-well Falcon plates at a density of 2.5×10^5 cells per well. Cells were maintained and counted every day after trypsinization. For measurements of [³H]thymidine incorporation, cells were plated in 24-well Falcon plates at a density of 0.5×10^5 cells per well and cultured in the presence of [³H]thymidine (1 mCi/ml) and increasing concentrations of rapamycin for the indicated times. The [³H]thymidine incorporation was measured by liquid scintillation counting. Ribosomal proteins were isolated from either mouse liver or MEFs, and the level of *in vivo* S6 phosphorylation was examined by two-dimensional polyacrylamide electrophoresis as previously described (Olivier *et al.*, 1988).

Polysome profiles and Northern blot analysis

Cytoplasmic extracts for polysome fractionation were prepared as already described (Jefferies *et al.*, 1997). The extracts were run on a 17.1–51% sucrose gradient in an SW60 Beckman rotor at 56 000 r.p.m. for 58 min at 4°C. Each sample was then fractionated into 12 fractions. Isolation of RNA and Northern blot analysis of the fractions were performed as described previously (Jefferies *et al.*, 1994b).

Cloning of S6K2 cDNA

A search in the EST database (Lennon *et al.*, 1995) for p70^{s6k} homologs provided us with the partial sequence of three clones (accession Nos AA563175, clone 975227; AA396470, clone 569218; and AA103535, clone 556081). The first clone showed similarity with the 5' end of p70^{s6k}, whereas the other two clones had overlapping sequence and were similar to the 3' end of p70^{s6k}. This sequence information was used to design two primers complementary to the predicted ends of a putative p70^{s6k} homolog. The sequence of the forward primer was 5'-CAG-AAGCTTATCTCCGAGGAGGACCTGGCGGCCGTATTTGATTTAG-ACTTGG and that of the reverse primer 5'-ATAAGAATCGGGC-CGCTGCAGTTCTCACAGCTGCCCTCTCTTCTCTATTCTCCTA-ACG. A 1.5 kb fragment was generated by PCR using these primers and cDNA from a mouse muscle library as a template (Stratagene). The 1.5 kb fragment was then amplified with the same reverse primer and the forward primer 5'-AGCTCGCGAGGATCCGTGGTTATGGAGCA-GAAGCTTATCTCCGAGG. The nested PCR introduced the sequence corresponding to the myc 9E10 epitope (Evan *et al.*, 1985) after the initiation codon of S6K2. The myc-S6K2 fragment was subcloned into the *Bam*HI and the *Not*I sites of a human cytomegalovirus promoter-driven expression vector and sequenced. Clone 975227 was obtained from American Type Culture Collection (ATCC) and entirely sequenced. The sequence of clone 975227 and the myc-S6K2 sequence were compared, and the sequence of the S6K2 open reading frame was registered in the DDBJ/EMBL/GenBank database under the accession No. AJ007938.

Antibody production

The peptide MAAVFDLDLEETEEGSEGEPEFSPADV, comprising the first 28 amino acids of S6K2, was coupled to a C-terminal KKC peptide, conjugated to keyhole limpet hemocyanin and used to produce rabbit polyclonal antiserum (Neosystem, Strasbourg, France).

Transient transfections, immunoprecipitation, immunoblotting and kinase assays

Human embryonic kidney 293 cells were maintained, transfected by a calcium phosphate precipitation method and stimulated as previously described (Dennis *et al.*, 1996). Protein concentrations were measured using the BioRad D/C protein assay. For Western blot analysis, either 50 μ g of protein extract from thymus or 20 μ g of protein extract from cells was resolved by SDS-PAGE before transfer onto an Immobilon P membrane (Millipore). Endogenous p70^{s6k} was monitored with either the M1 or M5 antibody as previously described (Lane *et al.*, 1992). Expression of the epitope-tagged S6K2 was detected with the monoclonal 9E10 antibody as previously described (Ming *et al.*, 1994). Expression levels were quantified using fluorimetry (Molecular Dynamics) and ImageQuant software (Molecular Dynamics). Kinase activity assays and quantitation of results were carried out as previously described (Pullen *et al.*, 1998).

Chromatography

Protein extracts were prepared and fractionated by Mono S cation-exchange chromatography as previously described (Lane and Thomas, 1991). Briefly, cells were washed twice with ice-cold extraction buffer [15 mM pyrophosphate, pH 6.8, 5 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)], scraped from the culture dish and homogenized with 12 strokes of a Dounce homogenizer. To remove cell debris, homogenates were first spun at 8000 *g* for 10 min at 4°C followed by ultracentrifugation at 340 000 *g* for 1 h at 4°C. Supernatants were filtered through a 0.22 μ m filter and loaded onto the Mono S column, pre-equilibrated with Mono S buffer (50 mM MOPS, pH 6.8, 1 mM EDTA, 10 mM pyrophosphate, 1 mM DTT, 1 mM benzamidine and 0.1% Triton X-100). The column was washed with Mono S buffer and developed with a 15 ml linear salt gradient up to 0.5 M NaCl, at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and assayed for S6 kinase activity at a 1:10 dilution (Lane and Thomas, 1991). For immunoprecipitation and Western blot analysis, fractions containing S6 kinase activity were pooled, diluted with 15 vols of peptide buffer (20 mM diethanolamine, pH 8.5, 1 mM EDTA, 1 mM benzamidine, 1 mM DTT, 15 mM pyrophosphate, 0.1% Triton X-100) and concentrated on a 50 μ l affinity column having as a solid phase S6 peptide (20 residue peptide S6_{228–249}) coupled to activated CH-Sepharose 4B (Ferrari, 1998). The column was then washed with 6 vols of peptide buffer containing 0.2 M NaCl, and proteins were eluted with 200 μ l of elution buffer (20 mM Bis-Tris, pH 6.3, 1 mM EDTA, 1 mM benzamidine, 1 mM DTT, 15 mM pyrophosphate, 0.5 M NaCl, 0.1% Triton X-100). A 50 μ l aliquot of the eluate was analyzed on Western blots using the anti-S6K2 antibody described above. A second 10 μ l aliquot was immunoprecipitated with the S6K2 antibody, and S6 kinase activity of the immune complex was assayed as described (Lane *et al.*, 1992).

RNA extraction and RNase protection assay

Total RNA from mouse tissues and cultured cells was extracted as previously described (Chomczynski and Sacchi, 1987). To obtain an S6K2-specific probe, the last 171 nucleotides of the S6K2 coding sequence were amplified by PCR using primers that introduced a *Bam*HI and an *Eco*RI site, respectively, at the 5' and 3' ends. The PCR fragment was subcloned into the *Bam*HI and the *Eco*RI sites of pBluescriptKS. The resulting plasmid was then linearized with *Bam*HI and served as a template to generate, by *in vitro* transcription with T3 polymerase, a radiolabeled antisense S6K2 RNA probe of 243 nucleotides. The S6K2 probe (10^5 c.p.m.) was added to 10 μ g of total RNA and dried in a speed-vac. RNA was resuspended in 10 μ l of hybridization solution (40 mM PIPES, pH 6.7, 400 mM NaCl, 1 mM EDTA and 80% formamide), incubated for 3 min at 95°C and for 16 h at 45°C. Samples were then incubated for 1 h at 28°C with 100 μ l of RNase solution (10 mM Tris, pH 7.5, 200 mM NaCl, 5 mM EDTA, 100 mM LiCl, 30 μ g/ml RNase A and 6 U/ml RNase T1). RNase digestion was terminated by adding 2 μ l of 10% SDS and 2 μ l of 20 mg/ml proteinase K. After incubation at 37°C for 15 min, RNA was extracted with phenol/chloroform and precipitated with ethanol using 20 μ g of tRNA as carrier. The RNA pellet was washed with 70% ethanol, resuspended in formamide loading buffer and loaded on a 5% polyacrylamide gel containing 8 M urea. At the end of the run, the gel was dried and analyzed by PhosphorImager (Molecular Dynamics).

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